

## Autonomic Innervation of Intra- and Extra-Cranial Arteries in the Amphibia

TAKASUKE TAGAWA, ATSUSHI ISHIKAWA, WANAN CHUAN SU  
and KIYOSHI OKAMOTO

*Department of Anatomy, Fukuoka University School of Medicine,  
Jonan-ku, Fukuoka 814-01, Japan*

**ABSTRACT**—The pattern of adrenergic and cholinergic innervation in the intra- and extra-cranial arteries of the internal carotid and vertebro-basilar systems was investigated using histochemical techniques in three amphibian species, the Japanese toad, the clawed toad and the leopard frog. As a common feature among these three anuran species, the adrenergic and cholinergic innervation density in the major cerebral artery system was low when compared with the extra-cranial internal carotid artery. In the Japanese toad the adrenergic and cholinergic nerve supply to the two cerebral artery systems was more predominant in the internal carotid system than in the vertebro-basilar system. It was similar in the leopard frog. Adrenergic nerve plexuses in the major cerebral arteries in the Japanese toad were dense compared with those in the leopard frog, but the density of cholinergic nerve plexuses was quite similar in both species. In the clawed toad the nerve fibers showing catecholamine fluorescence or acetylcholin-esterase (AChE) activity were not observed in any pial arteries, except for very few aminergic and AChE-positive fibers in the cerebral carotid artery. The direct innervation by the adrenergic and cholinergic nerves of central origin was demonstrated in the intracerebral small vessels of the clawed toad.

### INTRODUCTION

In mammalian cerebral vasculature, dual innervation by both sympathetic adrenergic and parasympathetic cholinergic fibers has been found histochemically [1-6]. Pharmacological and physiological studies have also shown vasomotor roles of these two populations of nerves on the cerebral vasculature [5-10]. In a previous series of histochemical studies in our laboratory, a unique aminergic and cholinergic innervation has been found in the cerebral blood vessels in a variety of submammalian species [11-15]. In the bullfrog, the major cerebral arteries are innervated only by the adrenergic fibers [14]. Besides this report, the innervation of amphibian cerebral vasculature has not been intensively studied using histochemical techniques. Here we report the distribution of aminergic and cholinergic nerves surrounding the cerebral blood vessels of three anuran species

other than bullfrog.

### MATERIALS AND METHODS

Thirty clawed toads (*Xenopus laevis*), 20 Japanese toads (*Bufo japonicus*) and 30 leopard frogs (*Rana nigromaculata*) were used in this study. The Falck-Hillarp formaldehyde fluorescence technique [16] and the direct coloring thiocholine method [17] were used for demonstrating the aminergic and cholinergic nerve fibers, respectively.

The animals were anesthetized with ethyl ether, perfused through the aorta with Ringer's solution and decapitated. For whole-mount preparations, the common carotid, intra-cranial carotid, extra-cranial carotid and cerebral arteries were carefully dissected out from the cervical regions and brain respectively. The materials were either stretched over nonfluorescent glass slides, transferred to a desiccator and vacuum dried over  $P_2O_5$  for 1 hr, or fixed with 4% buffered formaldehyde (pH 7.2) for 30 min at 4°C. For sectioning, small blocks of

brain tissue were quickly frozen in isopentane chilled with dry ice, and 15  $\mu\text{m}$  sections were cryostat sectioned, mounted on glass slides, and fixed with 4% cold formaldehyde for 30 min. Other blocks were freeze-dried.

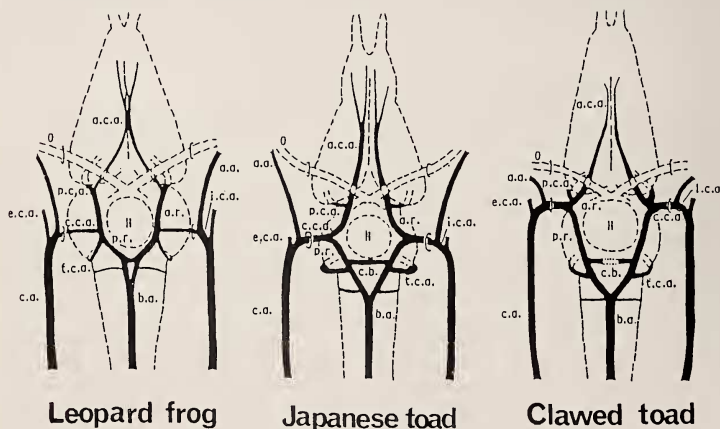
For demonstration of aminergic nerves, the air-dried materials and freeze-dried blocks were treated for 1 hr at 80°C with formaldehyde vapor from paraformaldehyde. The blocks were then infiltrated with paraffin and cut into 15  $\mu\text{m}$  sections. For cholinergic nerves, the whole-mount preparations fixed in formaldehyde and cryostat sections were maintained in Karnovsky's medium without acetylthiocholine iodide for 30 min at 4°C and then incubated in the complete medium, containing  $2 \times 10^{-4}$  M tetraisopropylpyrophosphoramide as a nonspecific cholinesterase inhibitor, for 1 to 5 hr at 20°C.

## RESULTS

A schematic illustration of the cerebral artery systems in the leopard frog, Japanese toad and clawed toad is shown in Figure 1. A characteristic feature of their cerebral vascular anatomy is the well-developed posterior cerebral artery (PCA) arising from the anterior ramus (AR), with an ill-developed middle cerebral artery, regarded as a small branch of the anterior cerebral artery (ACA). In addition, the anterior communicating artery is filamentous or even completely absent, resulting in an incomplete circle of Willis in most cases.

### *Aminergic innervation*

Dense plexuses of catecholaminergic nerve fibers, composed of thin fibers in a complicated meshwork, were found throughout the walls of the common and extra-cranial internal carotid arteries



①

FIG. 1. Diagrams of the arterial supply to the ventral surface of the brain in three species of amphibia. O, optic nerve; H, hypophysis; c.a., common carotid artery; i.c.a., internal carotid artery; e.c.a., external carotid artery; o.a., ophthalmic artery; c.c.a., cerebral carotid artery; a.r., anterior ramus; p.r., posterior ramus; p.c.a., posterior cerebral artery; a.c.a., anterior cerebral artery; t.c.a., tecto-cerebellar artery; b.a., basilar artery; c.b., posterior communicating branch.

(COCA, eICA) of the Japanese toad and leopard frog (Fig. 2). Nerve plexuses in the clawed toad COCA and eICA also demonstrated a plexiform appearance, but appeared to be less rich, and were organized in an elongated meshwork along the vascular axis (Fig. 3).

In the major cerebral arteries of the internal

carotid system (ICS) of the Japanese toad, the cerebral carotid artery (CCA) and AR were furnished with well-developed plexuses of adrenergic nerve fibers which were similar in density and meshwork construction to those seen in the COCA and eICA (Fig. 4). The PCA and ACA, were also richly supplied with adrenergic nerve fibers. In the

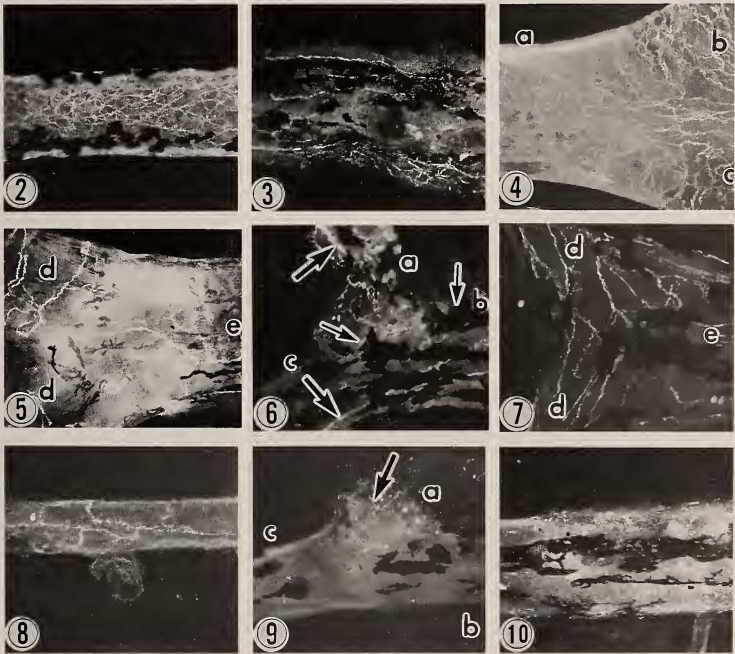


FIG. 2. Adrenergic innervation of common carotid artery in leopard frog.  $\times 108$

FIG. 3. Adrenergic innervation of common carotid artery in clawed toad.  $\times 108$

FIG. 4. Adrenergic innervation of cerebral carotid artery (a), anterior ramus (b) and posterior ramus (c) in Japanese toad.  $\times 108$

FIG. 5. Adrenergic innervation of posterior ramus (d) and basilar artery (e) in Japanese toad.  $\times 108$

FIG. 6. Adrenergic innervation of cerebral carotid artery (a), anterior ramus (b) and posterior ramus (c) in leopard frog. Arrows indicate the melanocyte.  $\times 108$

FIG. 7. Adrenergic innervation of posterior ramus (d) and basilar artery (e) in leopard frog.  $\times 108$

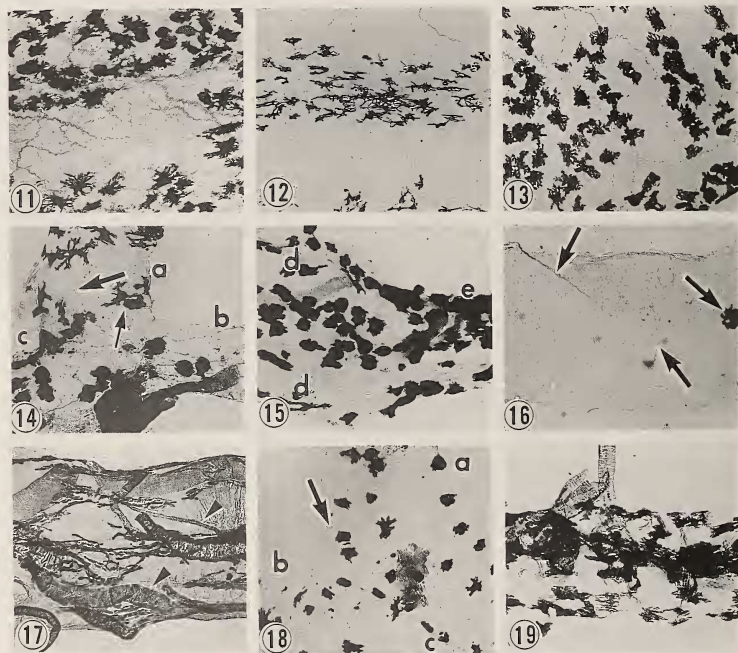
FIG. 8. Adrenergic innervation of anterior cerebral artery in leopard frog.  $\times 108$

FIG. 9. Adrenergic innervation of cerebral carotid artery (a), anterior ramus (b) and posterior ramus (c) in clawed toad. Adrenergic fibers (arrow) are indicated in the cerebral carotid artery.  $\times 108$

FIG. 10. Adrenergic innervation of basilar artery in clawed toad.  $\times 108$

major cerebral arteries of the vertebro-basilar system (VBS), the density of nerve plexuses was not so high as in the ICS; a moderate number of adrenergic nerve fibers formed wide networks along the walls of the PR and basilar artery (BA) (Fig. 5).

The density of adrenergic innervation in the cerebral artery system of the leopard frog was low compared with the Japanese toad: nerve plexuses of the CCA, AR, PCA, PR and distal portion of the BA demonstrated approximately the same density and distribution as those observed in the



- FIG. 11. Cholinergic innervation of common carotid artery in leopard frog.  $\times 108$   
 FIG. 12. Cholinergic innervation of common carotid artery in Japanese toad.  $\times 108$   
 FIG. 13. Cholinergic innervation of common carotid artery in clawed toad.  $\times 108$   
 FIG. 14. Cholinergic innervation of cerebral carotid artery (a), anterior ramus (b) and posterior ramus (c) in leopard frog. Thick arrow indicates the cholinergic fibers and thin arrow, the melanocyte.  
 FIG. 15. Cholinergic innervation of posterior ramus (d) and basilar artery (e) in leopard frog.  $\times 108$   
 FIG. 16. Cholinergic innervation of cerebral carotid artery in Japanese toad. Thick arrows indicate the cholinergic fibers and thin arrow, the melanocyte.  $\times 108$   
 FIG. 17. Cholinergic innervation of basilar artery and its small branches in Japanese toad. Relatively heavy AChE-activities on the walls of the small arteries are indicated by arrowheads.  $\times 108$   
 FIG. 18. Cholinergic innervation of cerebral carotid artery (a), anterior ramus (b) and posterior ramus (c) in clawed toad. AChE-positive fibers (arrow) are indicated in the cerebral carotid alone.  $\times 108$   
 FIG. 19. Cholinergic innervation of basilar artery in clawed toad.  $\times 108$



Japanese toad PR and BA (Figs. 6 and 7). The ACA and proximal portion of the BA of this frog were very poorly supplied by or sometimes devoid of adrenergic nerve fibers (Fig. 8). In the clawed toad, no adrenergic nerve fibers were demonstrated in the pial arteries, except for only a few fluorescent fibers in the CCA (Figs. 9 and 10).

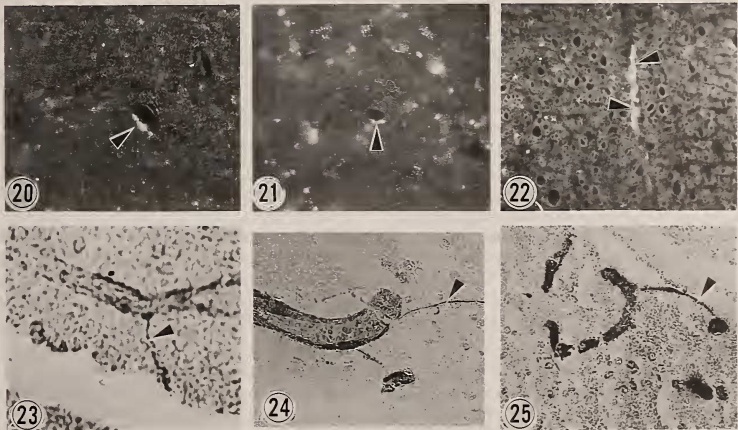
#### Cholinergic innervation

The COCA and eICA of the leopard frog possessed dense cholinergic nerve plexuses with a similar density and distribution to the adrenergic nerve plexuses (Fig. 11). Cholinergic nerve plexuses in the Japanese toad and the clawed toad COCA and eICA were also well organized, but they were more course than those in the leopard frog (Figs. 12 and 13).

The pattern of cholinergic innervation in the major cerebral arteries of the leopard frog corresponded well with the pattern of adrenergic innervation. A moderate number of nerve fibers positive for acetylcholinesterase (AChE) were found along the walls of the CCA, AR, PCA, PR

and distal portion of the BA (Figs. 14 and 15). The ACA and proximal portion of the BA received only a scarce supply of nerve fibers, with a low AChE activity. In the Japanese toad, the cholinergic nerve fibers surrounding the major cerebral arteries were distinctly fewer than the adrenergic nerves. Nerve fibers with very low AChE activity were sparsely distributed along the walls of the CCA, AR and PR (Fig. 16). In the remaining major cerebral arteries, AChE-positive nerve fibers were few or completely absent (Fig. 17). In the clawed toad, the distribution pattern of cholinergic nerve fibers was essentially the same as that of adrenergic nerve fibers and only a few AChE-positive fibers were observed in the CCA alone (Figs. 18 and 19).

It was apparent from the cross sections (Figs. 20–25) that capillaries and arterioles within the brain parenchyma are directly innervated by adrenergic and cholinergic nerve fibers. The direct adrenergic and cholinergic innervations of intraparenchymal small blood vessels were found in all of the species examined, and were often encoun-



FIGS. 20–25. Cross sections of capillaries and arterioles in the brain parenchyma. Adrenergic fibers (arrowhead) in the vessel wall in leopard frog (Fig. 20.  $\times 150$ ), Japanese toad (Fig. 21.  $\times 150$ ) and clawed toad (Fig. 22.  $\times 108$ ). Cholinergic fibers (arrowhead) running with the small blood vessels in leopard frog (Fig. 23.  $\times 200$ ), Japanese toad (Fig. 24.  $\times 200$ ) and clawed toad (Fig. 25.  $\times 200$ ).

tered in the blood vessels supplying the diencephalon.

### DISCUSSION

In mammals, the eICA, a major artery supplying blood to the cerebral circulation, has sparse adrenergic and cholinergic nerve plexuses, but the plexuses become dense rapidly as the ICA enters the cranial cavity [18]. In amphibian species studied, thus far ([14] and present findings) the eICA is more densely innervated by both adrenergic and cholinergic nerve fibers than the major cerebral arteries, or the mammalian eICA. Further, the innervation density of these two types of nerves in the amphibian eICA is high as compared with that in the corresponding artery of mammals. This suggests the significance of neurogenic vasomotor control in the amphibian eICA.

In our previous study, rich adrenergic innervation with no AChE-positive fibers has been found in the cerebral arteries of the bullfrog [14]. This innervation pattern cannot be regarded as a common feature of amphibian cerebrovascular innervation, since the present study has shown the presence of AChE-positive fibers in the major cerebral arteries of the clawed toad, Japanese toad and leopard frog.

In various mammalian species, adrenergic and cholinergic nerve fibers richly innervate the major cerebral arteries with approximately the same density [1-6, 19]. The dual innervation by adrenergic and cholinergic nerves has also been demonstrated in the cerebral arteries of the turtle [11], snake [12] and domestic fowl [13]. Unlike the mammals, the cerebrovascular innervation of these submammalian species is characterized by an unbalanced innervation with fewer cholinergic nerve fibers. In amphibians, although the density and distribution of cerebral perivascular adrenergic and cholinergic nerve fibers varies considerably from species to species, the adrenergic innervation usually predominates. Furthermore, the major cerebral arteries of the lamprey are innervated solely with serotonergic nerve fibers [15]. From these histochemical studies ([11-15] and present findings), we could assume an evolutionary trend that

aminergic fibers appeared earlier than cholinergic fibers in cerebral vascular autonomic innervation. In visceral and cardiovascular autonomic innervation, however, it was oppositely argued that cholinergic fibers appeared earlier than aminergic fibers [20].

It is generally accepted in various vertebrates that sympathetic adrenergic nerves surrounding the major cerebral arteries penetrate into the brain parenchyma along vascular branches to innervate the small intracerebral blood vessels [1-4, 11-14, 18, 19]. The innervation of small blood vessels within brain parenchyma by peripheral cholinergic nerves has also been confirmed in bats [19]. In addition, the existence of central adrenergic and cholinergic innervation has been demonstrated histochemically or ultrastructurally in the intracerebral capillaries and arterioles of laboratory mammals, with a combination of ganglionectomy or denervation technique [21-27]. Similar findings have also been noted in some submammalian species [11-15]. In the present study, dual adrenergic and cholinergic innervation has been observed in the small arteries and capillaries supplying the brain parenchyma, especially the diencephalon, in all the anuran species examined. Although we did not approach to the origin of these fluorescent and AChE-positive nerve fibers with ganglionectomy and denervation treatment in the present study, the absence of aminergic and AChE-positive nerve fibers in the major cerebral arteries of the clawed toad indicates that the aminergic and AChE-positive fibers innervating the intraparenchymal small vessels of this toad are of central origin.

A clear species difference is observed in the innervation density of adrenergic and cholinergic nerves in the amphibian cerebral artery system. This may reflect the uniqueness of adrenergic and cholinergic mechanisms operating in the cerebral circulation of respective amphibian species. Besides these classical neurotransmitters, our preliminary immunohistochemical study has also demonstrated the existence of nerve fibers containing vasoactive peptides, such as substance P, neurokinin A, calcitonin gene-related peptide and vasoactive intestinal polypeptide, in the major cerebral arteries of the bullfrog (Tagawa *et al.*, unpublished

data). The functional involvement of catecholamines, acetylcholine and different kinds of vasoactive peptides within cerebral perivascular nerves is not known in amphibia, and even in laboratory mammals it is still a matter of speculation. In order to explore the neurogenic control mechanisms in the amphibian cerebral circulation, it is necessary to resolve the interaction among these neurotransmitters and putative neurotransmitters.

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